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Table of Contents

INTRODUCTION

Early detection is the most significant means for reducing the morbidity and mortality due to breast cancer. In addition to mammography and physical examination, sensitive molecular techniques may also be used to detect early stage breast cancer. Evidence suggests that breast cancer cells are released into the circulation at an early stage of the disease (1). A recent study reported that 95% of early stage breast cancer patients had circulating cells that could be detected immunohistochemically with a nonspecific epithelial antibody (2). In addition, mammaglobin, the most breast-specific gene known, was detected in circulating tumor cells in the blood from 20 of 32 metastatic breast cancer patients (3). These intriguing results clearly demand validation with studies that can sensitively and specifically detect circulating breast cancer cells. We hypothesize that sensitive molecular detection of cancer cells in peripheral blood using novel breast-specific genes will provide a screening test that can be used independently or in concert with mammography and physical examination to more accurately detect early stage breast cancer.

BODY

The goal of this project was to detect circulating cancer cells in the blood from 200 patients who had suspicious mammograms and breast tissue biopsies using sensitive molecular detection of breast-specific tumor markers. This study determined the clinical significance of circulating cancer cells in a select population of patients. We developed an immunomagnetic cell capture technique that is coupled with RT-PCR for detection of breast cancer cells in blood and bone marrow (4). This method is extremely sensitive and is capable of detecting one breast cancer cell in 10^7 nucleated cells in a model test system using MDA-MB361 breast cancer cells (4). We used unique, breast-specific genes including mammaglobin, B305D-C form, B726P, and γ-aminobutyrate type A receptor π subunit (GABA A_π; i.e., B899P) with complementary expression in 100% of breast cancers (3).

Patient accrual:

We collected 20 milliliters of blood from 180 patients who had suspicious mammograms between July 16, 2001 and October 30, 2002. Although the funding from the Department of Defense ended September 30, 2002, we are continuing to collect blood samples to reach the accrual number of 200 patients, which is anticipated by the end of December, 2002. Our own institutional funds will be used to complete the analyses of these remaining samples. Samples from 17 of the 180 registered patients were removed from the study (study cancellations) due to a reported prior history of invasive cancer or because no breast tissue biopsies were performed (e.g., fine needle aspirations or ultrasounds were performed). All patients were informed of the investigational aspects of this study and provided written consent in accordance with institutional and federal guidelines. All blood samples were assigned a unique identification number. Approximately 18 milliliters of blood were analyzed from each sample. We finished the molecular analyses of the samples from 156 eligible patients. The analyses of the remaining seven samples are in progress. The patient population is defined in **Table 1**. Seventy percent of the patients had benign breast disease. We classified the breast biopsies of these patients as negative because most had micro-calcifications, fibrocystic and/or non-fibrocystic changes. As expected, approximately 24% of 156 eligible patients had invasive breast cancer and 6% had ductal carcinoma in situ (DCIS). Unfortunately, we had to exclude a low percentage of samples due to technical difficulties that arose during processing. Actin levels below 0.1 pg were observed in 3% and poor separation of epithelial cells from the Epithelial Enrich Dynal beads was observed in 9% of the cases.

Table 1. Patient cohort.

Study Population	N
Total patients enrolled	180
Study cancellations	17
Completed analyses	156
Not completed analyses	7
Biopsy Result	
Benign Breast Disease (e.g., micro-calcifications or fibrocystic changes)	109
Exclude samples-technical difficulties	15
Ductal Carcinoma In Situ (DCIS)	9
Exclude samples-technical difficulties	2
Invasive Breast Cancer	38
Exclude samples-technical difficulties	2

Technical difficulties include actin levels of less than 0.10 pg and/or poor separation of the epithelial cells from the magnetic Dynal beads.

The clinicopathological characteristics of the primary, invasive breast tumors are shown in **Table 2**. Thirty-one percent of the 36 patients with invasive breast cancer were less than 51 years of age, 39% of the breast cancer patients were between 50 and 61 years of age, and 31% of the breast cancer patients were greater than 60 years of age. The majority of the breast cancers were invasive ductal carcinomas (72%), and the remaining cancers were either invasive lobular (8%) or invasive tubular (3%) or a combination of ductal and lobular (14%) or ductal and tubular (3%). Twenty-five percent of the 36 tumors were grade I, 39% were grade II, and 36% were grade III. The majority of the tumors were T1's (53%), 36% were T2's and the remaining 11% were T3's. Sixty-seven percent of the 36 breast cancer patients had no nodal involvement, whereas 33% of the patients had nodal involvement. The majority of the tumors were hormone receptor positive (ER+: 78%; PR+: 75%), and 19% and 22% of the tumors were ER- and PR-, respectively. Only 11% of the 36 tumors were Her2Neu positive (indicated by a score of 2+ or 3+), 33% were negative for Her2Neu (indicated by a score of 0 or 1+), and the Her2Neu status was not determined in 58% of the cases because the majority of these cases were node-negative.

Table 2. Clinicopathological characteristics of 32 primary breast cancers

Patient #	Age	Histology	Grade	Tumor Size	Nodal Status	Her2	ER	PR
95	47	IL	I	1.1cm	N-	ND	+	+
96	70	IL	II	6 cm	N+	1+	+	+
97	62	ID	III	1.8 cm	N-	ND	+	+
98	81	ID	III	4.0 cm	N+	1+	+	+
99	44	IT	I	3 mm	N-	ND*	ND*	ND*
100	41	ID	III	2.8 cm	N-	ND	+	+
101	73	ID	III	2.2 cm	N-	ND	+	+
102	60	IL	II	2.4 cm	N-	0	+	+
103	79	ID	I	0.6 cm	N-	ND	+	+
104	43	ID	II	5.5 cm	N+	2+/3+	+	+
105	56	ID	III	2.4 cm	N+	1+	-	-
106	60	ID	II	6 mm	N-	ND	+	+
107	69	ID/IL	II	2.5 cm	N+	2+/3+	+	+
108	55	ID	II	1.3cm	N-	ND	+	+
109	56	ID	III	2.2 cm	N-	ND	-	-
110	49	ID/IL	II	5.8 cm	N+	ND	+	+
111	60	ID	I	1.5 cm	N-	FISH (0)	+	+
112	73	ID/IL	II	0.7 cm	N-	ND	+	+
113	53	ID	II	2.4 cm	N+	0	+	+
114	49	ID	III	2.2cm	N+	0	-	-
115	54	ID	I	1.0 cm	N-	ND	+	+
116	57	ID	III	2.7 cm	N+	0	-	-
117	66	ID	III	5.7 cm	N+	0	-	-
118	55	ID	II	1.0 cm	N-	ND	+	+
119	51	ID	III	1.4 cm	N-	ND	+	+
120	37	ID	III	2.3 cm	N-	ND	-	-
121	50	ID	III	2.7 cm	N-	ND	-	-
122	58	ID/IT	I	0.8 cm	N-	FISH (0)	+	+
123	73	ID	I	0.9 cm	N-	ND	+	+
124	50	ID	II	1.1 cm	N-	ND	+	+
125	43	IL/ID	I	3.0 cm	N+	3+	+	-
126	47	ID/IL	II	2.0 cm	N-	ND	+	+
127	65	ID	I	1.9 cm	N-	0	+	+
128	47	ID	II	1.7 cm	N+	3+	+	+
129	65	ID	II	1.6 cm	N-	ND	+	+
130	51	ID	III	1.3 cm	N-	0	+	+

Positive nodal status (+) is determined as having at least one positive axillary node detected at the time of primary surgery. Her2 status was determined by immunohistochemical staining using HercepTest™ unless otherwise indicated. A positive ER or PR status (+) is determined as having any neoplastic cell immunohistochemically stained for ER or PR. IL: invasive lobular carcinoma; ID: invasive ductal carcinoma; IT: invasive tubular; ND: not determined; ND*: not determined-insufficient tissue.

Molecular detection methods and progress:

1). Isolation of mononuclear cells from the blood using density gradient centrifugation.

2). Immunomagnetic capture of epithelial cells.

We isolated mononuclear cells and captured the epithelial cells from all the 180 collected blood samples within one day of each blood draw. We isolated mononuclear cells by density gradient centrifugation using an Accuspin Histopaque-1077 system from Sigma Aldrich (St. Louis, MO). The epithelial cells were isolated by immunomagnetic capture using the monoclonal antibody, BER-EP4, and the magnetic Dynabeads Epithelial Enrich kit (Dynal A.S., Oslo, Norway). The BER-EP4 antibody recognizes two glycoproteins on the surface and in the cytoplasm of all epithelial cells except the superficial layers of squamous epithelia, hepatocytes, and parietal cells (5).

3) & 4). Isolation of mRNA and reverse transcription of mRNA.

The isolation and reverse transcription of mRNA was completed for the samples from 156 eligible patients. We isolated mRNA using the mRNA Direct kit and Dynabeads Oligo $(dT)_{25}$ (Dynal A.S., Oslo, Norway). We then reverse transcribed the mRNA (attached to the Dynabeads Oligo $(dT)_{25}$) using AMV reverse transcriptase (Roche Diagnostics, Indianapolis, IN) and random hexamers (Roche Diagnostics, Indianapolis, IN), which resulted in a first strand cDNA that is covalently attached to the beads. This solid phase cDNA library was stored at 4°C.

5). Second strand synthesis using gene-specific, forward primers.

The solid-phase cDNA library was then used repeatedly with gene-specific, forward primers and Taq polymerase (Roche Diagnostics, Indianapolis, IN) to synthesize second strand cDNA (6, 7), which served as a template for quantitative real-time PCR (8,9). Second strand syntheses for mammaglobin, B305D-C form, and B726P, B899P, cytokeratin-19 (CK-19), and β-actin was completed for all the samples from 156 eligible patients.

6). Gene expression analyses using real time PCR .

The second strand cDNA from each sample (stored at -80°C) was amplified by fluorescent-based kinetic PCR with gene-specific primers using an ABI 7700 prism Taqman™ instrument (PE Biosystems, Foster City, CA). Circulating cells were detected using mammaglobin, B305D-C form, B726P, B899P, CK-19, and β-actin. The PCR assays using Taqman™ chemistries and real-time PCR (8, 9) for each gene were successfully developed in our laboratory. The sequences for the primers and fluorescent probes for B305D-C form, B726P, and B899P were obtained from Houghton and coworkers (4). Matching primers and fluorescent probes were designed for mammaglobin and CK-19 using the Primer Express Program (Applied Biosystems, Foster City, CA). The primers and probes for all the genes except β-actin were purchased from Integrated DNA Technologies (Coralville, IA). The β-actin primers and probe were obtained from a kit purchased from Applied Biosystems (Foster City, CA). Plasmids containing the gene of interest were constructed using the Original TA Cloning kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). These plasmids or oligonucleotides of the specific amplicons were used in the real-time PCR assays to generate standard curves to allow for quantification of gene expression. The presence of β-actin mRNA was used as an endogenous control in the samples to normalize the gene expression of mammaglobin, B305D, B726P, and GABA A_π. The primer and probe sequences for each gene are shown in **Table 3**.

Table 3. Taqman primer and probe sequences

Gene	Forward Primer	Position	Reverse Primer	Position	Probe	Position	Product Size (bp)
β-actin	TCACCCACACTG TGCCCATCTACGA	2141	CAGCGGAACCGC TCATTGCCAATGG	2411	ATGCCCCCCCCA TGCCATCCTGCGT	2171	295
Mamma-globin	AGAACTGCAGGG ATGGTGAGAA	367	ACATGTATAGCAGGT TTCAACAATTGT	481	CCAACTACGGATTGC TGCAAACCACA	391	114
CK-19	CCAGCGGCTCAT GGACAT	1121	CTTGGAGGCAGACAA ATTGTTG	1226	AGCAGGAGATTGCCA CCTACCGCA	1153	105
B305D -C Form	AAAGCAGATGGT GGTTGAGGTT	117906	CCTGAGACCAAATGG CTTCTTC	117975	ATTCCATGCCGG CTGCTTCTTCTG	117929	69
B726P	TCTGGTTTTCTCATT CTTTATTCATTTATT	235	TGCCAAGGAGCGGATT ATCT	326	CAACCACGTGACAAA CACTGGAATTACAGG	268	91
B899P	AAGCCTCAGAGTCC TTCCAGTATG	2090	AAATATAAGTGAAGA AAAAATTAGTAGAT	2192	AATCCATTGTATCTTAGA ACCGAGGGATTTGTTTAGA	2128	102

6). *Statistical Analyses.*

Sensitivity and specificity analyses were used to assess the discriminating ability of mammaglobin, B305D-C form, B726P, and B899P alone and in combination. We examined the role of these four genetic markers in discriminating among women with negative breast biopsies (benign breast disease; e.g., micro-calcifications and fibrocystic changes) and those with positive breast biopsies (invasive breast cancer). Receiver operator characteristic (ROC) curves were constructed to visualize the effect of altering the definition of positivity on sensitivity and specificity. A sample was said to be positive (to have invasive breast cancer) if its gene expression value was above the experimental cut-point. Based on the single gene results with moderately high discriminating power, any two genes were paired with one another to determine whether an increase in discriminating power after combination was observed. To do so, we examined a grid of values. Sensitivity and specificity analyses were performed around the final cut-points based on single gene results. Sensitivity and specificity were calculated for each gene combination, where a gene combination was said to be positive if at least one gene's copy number exceeded the cutpoint. The pairwise values that maximized both sensitivity and specificity were chosen to be the final cut-points.

Research Accomplishments

Gene expression in negative and positive breast biopsies.

Because CK-19 is a marker for epithelial cells, we examined its gene expression in the captured epithelial cells from the blood samples. We found that the majority (67%) of the blood samples from the positive invasive breast biopsies had greater than 45 copies of CK-19 per approximately 18 milliliters of blood analyzed (approximate average of CK-19 gene copies of normal healthy females). Nineteen percent of these samples had greater than 500 CK-19 copies per the 18 milliliters of blood. Whereas, only 47% of the blood samples from the negative breast biopsies had greater than 45 copies of CK-19 and only 4% of these samples had greater than 500 CK-19 copies per the 18 milliliters of blood analyzed. These results indicate that patients with invasive breast biopsies have increased number of circulating epithelial cells compared to patients with negative breast biopsies. Yet, a small percentage of these negative breast biopsy patients did have significant detectable levels of circulating epithelial cells.

To determine the discriminatory ability of mammaglobin, B305D-C form, B726P, and B899P, we compared their gene expression levels (normalized to β-actin) between samples from patients with negative and positive breast biopsies. Because preliminary results from our laboratory suggest that the baseline levels of mammaglobin and possibly B305D-C form, B726P, and B899P may change throughout the menstrual cycle of normal, healthy females, we determined gene expression threshold values that would optimally discriminate between the two types of biopsies using sensitivity and specificity analyses as described in the Statistical Methods Section. **Figures 1** and **2** illustrate the mRNA levels of mammaglobin and B305D-C form in captured epithelial cells from patients with positive and negative breast biopsies. As shown in **Figure 1**, mammaglobin at a threshold of 46.4 copies/pg β-actin, was detected in the captured epithelial cells from 14% of the 94 patients with negative breast biopsies (sample numbers 1-94) and from

56% of 36 patients with positive breast biopsies (samples numbers 95-130). Only two DCIS samples (sample numbers 131-137) were detected at the lower threshold of 27.8 copies/pg β-actin. As illustrated in **Figure 1**, this lower cutpoint provided an increased sensitivity (64%) and a decreased specificity (71%) compared to the higher cutpoint.

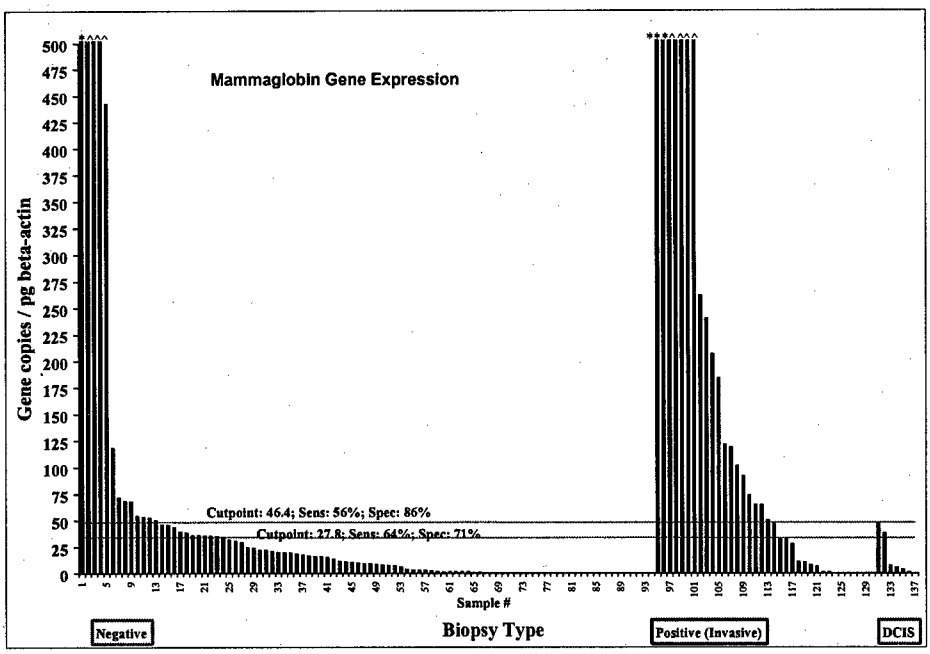

Figure 1. Mammaglobin Gene Expression in Biopsies. *: Mammaglobin levels greater than 500 gene copies/pg β-actin; ^: Mammaglobin levels greater than 1000 copies/pg β-actin.

As illustrated in **Figure 2**, B305D-C form at a threshold of 5.61 copies/pg β-actin, was detected in the captured epithelial cells from 21% of the 94 patients with negative breast biopsies and from 53% of the 36 patients with positive breast biopsies. At the lower threshold level of 2.43 copies/ pg β-actin, B305D-C form detected three patients with DCIS. As illustrated in **Figure 2**, this lower cutpoint provided an increased sensitivity (61%) and a decreased specificity (62%) compared to the higher cutpoint.

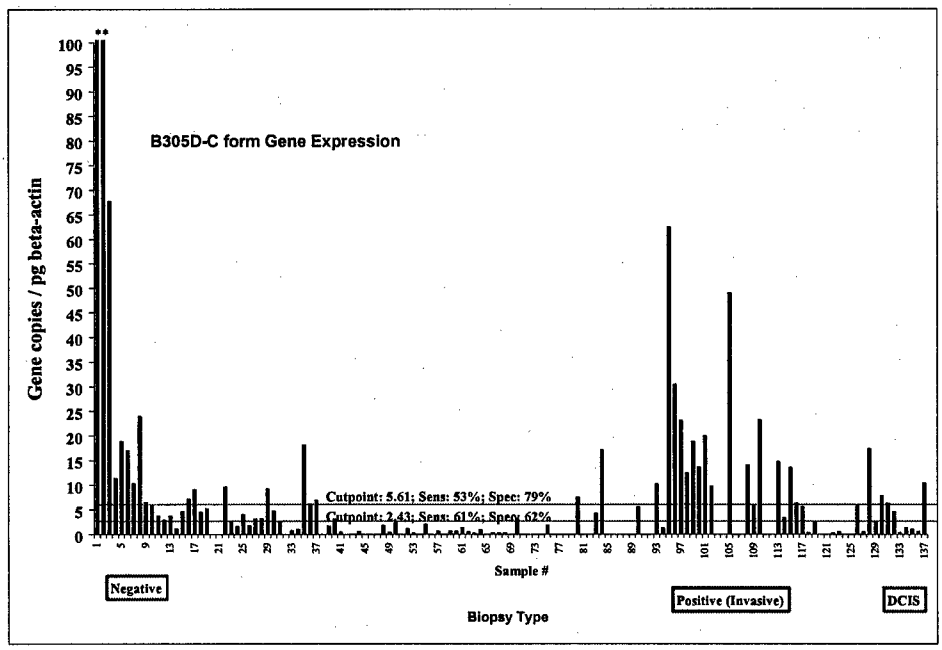

Figure 2. B305D-C Form Gene Expression in Biopsies. *: B305D-C form gene expression greater than 100 copies/pg β-actin.

Discriminatory ability of mammaglobin, B305D-C form, B726P, and B899P.

The optimal discriminatory powers of the unique, breast specific genes alone and in combination are described in **Tables 4 and 5**. Of the four genes examined, mammaglobin alone at a cutpoint of 27.8 copies/pg β-actin most accurately distinguished between patients with benign breast disease and invasive breast cancer with an optimum sensitivity and specificity of 64% and 71%, respectively. Specificity of mammaglobin alone dramatically increased to 86% and sensitivity slightly decreased to 56% at a cutpoint of 46.39 copies/pg β-actin. B726P alone fairly accurately discriminated between the two types of biopsies with an optimum sensitivity and specificity of 64% and 62%, respectively. B305D-C form alone also fairly accurately discriminated between the two types of biopsies with an optimum sensitivity and specificity of 61% and 62%, respectively. Specificity of B305D-C form dramatically increased to 79% and sensitivity slightly decreased to 53% at a B305D-C form cutpoint of 5.61 copies/pg β-actin.

Table 4. Individual Optimal Discriminatory Power of Mammaglobin, B305D-C Form, B726P, and B899P. Mamm: mammaglobin. Values in parentheses represent the corresponding confidence intervals.

Gene	Cut-point	Sensitivity	Specificity
MAMM	27.776	63.9 (46.2, 79.2)	71.3 (61.0, 80.1)
MAMM	46.394	55.6 (38.1,72.1)	86.2 (77.5,92.4)
B305DC	2.432	61.1 (43.5,76.9)	61.7 (51.1,1 71.5)
B305DC	5.612	52.8 (35.5, 69.6)	78.7 (69.1, 86.5)
B726P	0.874	63.9 (46.2, 79.2)	61.7 (51.1, 71.5)
B899P	0.301	50.0 (32.9, 67.1)	54.3 (43.7, 64.6)

Gene complementation.

As shown in **Figure 3**, complementary gene expression of mammaglobin and B305D-C form (at the indicated levels) was detected in the captured epithelial cells from 23% of the 94 patients with negative biopsies and in 72% of the 36 patients with positive breast biopsies. Although six mammaglobin-negative patients were detected with B305D-C form, B726P and B899P did not provide additional detection of circulating cancer cells from primary breast cancer patients (data not shown). Consequently, complementation of mammaglobin with B305D-C form increased the sensitivity to 72% and provided a specificity of 76% (**Table 5**). In addition, both mammaglobin and B305D-C form detected 92% of the patients with node-positive disease. Seven of the 10 samples not detected by mammaglobin or B305D-C form contained low CK-19 levels (< 45 copies/18 milliliters blood) indicating that our technique did not efficiently capture epithelial cells from those blood samples.

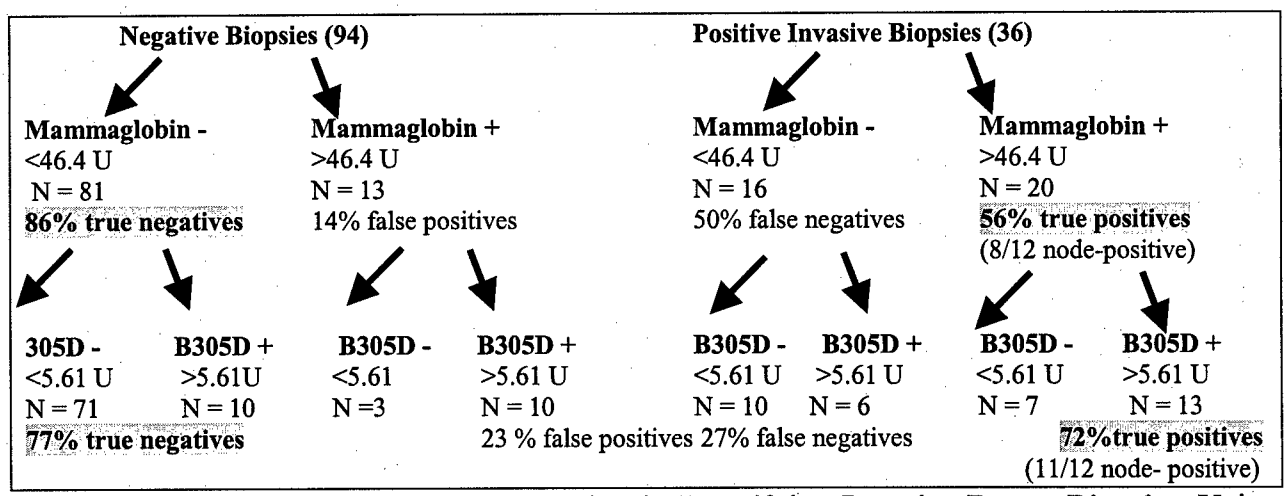

Figure 3. Complementary Gene Expression in Identifying Invasive Breast Biopsies. Units (U) are copies/pg β-actin.

Although complementation of mammaglobin with B726P provided fairly accurate discrimination between patients with negative and positive breast biopsies (70% sensitivity and 68% specificity), this combination did not perform as well as the complementation of mammaglobin with B305D-C form (**Table 5**).

Table 5. Discriminatory Power of Mammaglobin, B305D-C Form, B726P, and B899P in Combination. Mamm: mammaglobin. Values in parentheses represent the corresponding confidence intervals.

Gene	Cut-point	Gene	Cut-point	Sensitivity	Specificity
MAMM	27.776	**B305DC**	9.539	66.7 (49.0, 81.4)	68.1 (57.7, 77.3)
MAMM	46.394	**B305DC**	5.612	72.2 (54.8, 85.8)	75.5 (65.6, 83.8)
MAMM	31.865	**B726P**	3.0	66.7 (49.0, 81.4)	67.0 (56.6, 76.4)
MAMM	46.394	**B726P**	1.597	69.4 (51.9, 83.7)	68.1 (57.7, 77.3)
B305DC	2.432	**B726P**	2.923	66.7 (49.0, 81.4)	60.6 (50.0, 70.6)

Problems and recommended changes.

Due to unforeseen circumstances, we did not reach the anticipated accrual number of 200 patients for the September 30, 2002 grant end date. As stated in the October 2001 annual report, we initially planned to include the Mayo Clinic in Jacksonville, FL in this study with Dr. Edith Perez as co-principal investigator in Jacksonville. However, due to delays in IRB and HSSRB approvals, Mayo Clinic, Jacksonville did not participate in this study. In addition, other studies recruiting the same population base as this study were opened at our institution.

Several laboratories including ours have developed positive immunomagnetic techniques for detection of tumor cells in bone marrow and peripheral blood, and a 3-4 log tumor cell enrichment was demonstrated (3; 10, 11). However, some problems can occur with the positive selection technique. We observed difficulties in separating the captured epithelial cells from the Dynal epithelial beads in 9% of the 156 samples analyzed, which may have resulted in epithelial bead contamination in the mRNA isolation step. Although

genomic DNA contamination may have been possible at this separation step, mRNA was only transcribed at the RNA isolation step because we employed Oligo $(dt)_{25}$ beads. After the reverse transcription step, we further synthesized gene specific second strands. These steps and multiple washings tend to minimize genomic DNA contamination at the later stages of the protocol. However, genomic DNA contamination may have been responsible for the high gene expressions detected in the few negative breast biopsies (samples # 1-4 of figures 1-2).

Although the tumor cell purity registered by these positive enrichment techniques is superior to negative immunomagnetic separation, this negative technique enriches for tumor cells regardless of the epithelial antigen characteristics of the cells. Positive enrichment requires the presence of a particular antigen recognized by the immunobeads. Consequently, negative immunomagnetic separation may be more reliable in situations where micrometastatic tumor cells are poorly characterized and is well suited for immunocytochemical procedures. However, high tumor cell purity is more favorable for RT/PCR-based detections, since the risk of illegitimate expression of the epithelial target mRNA in rare hematopoietic cells is considerable (12-14).

If further funding is awarded for validation studies, some changes may be warranted. Although no signal was detected in our control samples containing no reverse transcriptase enzyme (indicating no genomic DNA contamination), primer sets designed to span intron/exon junctions and Dnase treatment of the samples will be employed. New primer and probe sets were designed recently for mammaglobin, B305D-C form, B726P, and B899P that spanned intron/exon junctions (15). Also, negative enrichment techniques (e.g., RosetteSep system by StemCell Technologies, Vancouver, Canada) will be compared to our current technique, and the more sensitive technique will be used. Results from previous studies demonstrated that the RosetteSep CD45 depletion system provided an increased sensitivity compared to the Dynal Epithelial Enrich beads (16-17). Sensitivity and specificity may possibly be increased with additional genes. We are in the process of exploring and examining good candidate genes.

KEY RESEARCH ACCOMPLISHMENTS

- Successful development of isolating circulating epithelial cells.
- Successful development of quantifying gene expression in the captured epithelial cells.
- Determined that circulating tumor cells are clinically significant.
- Detection of circulating cancer cells in the blood using a panel of genes that display complementary expression has the potential to be used as a sensitive screening tool for breast cancer.
    - Complementation of mammaglobin with B305D-C form in captured epithelial cells from the blood of a population of patients with suspicious mammograms provided a screening test to detect circulating tumor cells with sensitivity and specificity of 72% and 76%, respectively.
    - Complementation of mammaglobin with B305D-C form also detected 92% of the node-positive breast-cancer patients.
- Further increase of sensitivity and specificity is possible with additional genes (e.g., B511S).

REPORTABLE OUTCOMES

Preliminary results from this study were reported in abstract form at the 2002 Era of Hope Meeting in Orlando Florida sponsored by the Department of Defense (18). We anticipate submitting a manuscript from this study in late December of 2002 or early January 2003 to a highly respected journal such as Clinical Cancer Research.

CONCLUSIONS

The research supported by this Concept Award yielded results with significant implications regarding the early detection of breast cancer. These results suggest that sensitive molecular techniques can also be used alone or in combination with mammography and physical examination to more accurately detect early stage invasive breast cancer. Our results determined that circulating breast cancer cells are rarely detected in non-invasive breast cancer patients but are clinically significant in early stage invasive breast cancer patients as well as in node-positive patients. Using only two markers, we determined that circulating tumor cells were found in 72% of invasive breast cancer patients. Although the false-positive rate was 24%, simple modifications to our procedure (e.g., negative enrichment, intron/exon spanning primers, DNase treatment) may improve sensitivity and specificity. In addition, we anticipate inclusion of additional genes, such as B511S, which displayed good complementation with mammaglobin in primary breast tumors (4) will further increase the sensitivity and specificity of this potential screening test. If additional funding is granted, a screening test will be validated in future studies with an appropriate population of patients. If validated, this test would detect patients with breast cancer while decreasing the number of unnecessary biopsies and false negative mammographic results. Overall, the results from the research supported by this award suggest that detection of circulating cancer cells in the blood using a panel of genes that display complementary expression has excellent potential as a more accurate screening tool for breast cancer. The potential positive impact of a screening blood test for breast cancer cannot be overstated.

REFERENCES

1. Racila E, Euhus D, Weiss AJ, Rao C, McConnell J, Terstappen LW, and Uhr JW, Detection and characterization of carcinoma cells in the blood. *Proc. Natl. Acad. Sci. USA*. **95,** 4589-4594 (1998).
2. Krag DN, Beitsch PD, Moss TJ, Kusminsky RE, Feldman S, Carp NZ, Moffatt FL, Frazier TG, Gaskin TA, Shook JW, Harlow SP, and Weaver DL, Breast cancer cells in the blood. *Breast Cancer Res Treat.* **57**,120 (1999).
3. Roche PC, Kitzmann KA, Klee SM, Grant CS, and Ingle JN, Detection of breast cancer cell in bone marrow and peripheral blood with immunomagnetic capture and RT-PCR for mammoglobin. *Breast Cancer Res Treat.* **57**, 119 (1999).
4. Houghton RL, Dillon DC, Molesh DA, Zehentner BX, Xu J, Jiang J, Schmidt C, Frudakis A, Repasky E, Maltez Filho A, Nolasco M, Badaro R, Zhang X, Roche PC, Persing DH, and Reed SG, Transcriptional complementarity in breast cancer: application to detection of circulating tumor cells. *Mol Diagn.* **6**, 79 (2001).

5. Latza U, Niedobitek G, Schwarting R, Nekarda H, Stein H, Ber-EP4: New monoclonal antibody which distinguishes epithelia from mesothelia. J Clin Pathol; 43:213-219 (1990).
6. Raineri I, Moroni C, and Senn HP, Improved efficiency for single-sided PCR by creating a reusable pool of first-strand cDNA coupled to a solid phase. *Nucl. Acids Res.* **19,** 4010 (1991).
7. Fellmann F, Pretat J-L, and Fellmann D, Simplified protocol of solid-phase cDNA libraries for multiple PCR amplification. *Biotechniques* **21,** 766 (1996).
8. Holland PM, Abramson RD, Watson R, and Gelfand DH, Detection of specific polymerase chain reaction products by utilizing the 5' to 3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 7276 (1991).
9. Heid CA, Stevens J, Livak KJ, and Williams PM: Real time quantitative PCR. *Genome Res* **6,** 986-994, (1996).
10. Griwatz C, Brandt B, Assmann G, and Zanker KS, An immunological enrichment method for epithelial cells from peripheral blood. *J. Immunol. Methods* **183,** 251-265 (1995).
11. Naume B, Borgen E, Beiske K, Herstad T, Ravnas G, Renolen A, Thrane-Steen K, Trachsel S, Funderud S, and Kvalheim G, immunomagnetic techniques for the enrichment and detection of isolated breast carcinoma cells in bone marrow and peripheral blood. *J. Hematother* **6,** 103-113 (1997).
12. Krissmann M, Todt B, Schroder J, Gareis D, Muller KM, Seeber S, and Schutte J, Low specificity of cytokeratin 19 reverse-transcriptase-polymerase chain reaction analyses for detection of hematogenous lung cancer dissemination. *J. Clin Oncol* **13,** 2769-2775 (1995).
13. Eaton MC, Hardingham JE, Kotasek D, and Dobrovic A, Immunobead RT-PCR: a sensitive method for detection of circulating tumor cells. *Biotechniques* **22,** 100-105 (1997).
14. Zippelius A, Kufer P, Honold G, Kollermann MW, Oberneder R, Schlimok G, Riethmuller G, and Pantel K, Limitations of reverse transcriptase polymerase chain reaction analyses for detection of micrometastatic epithelial cells in bone marrow. *J. Clin Oncol* **15,** 2701-2708 (1997).
15. Zehentner BK, Dillon DC, Jiang Y, Xu J, Bennington A, Molesh DA, Zhan X, Reed SG, Persing DH, and Houghton RL, Application of a multigene reverse transcription-PCR assay for detection of mammaglobin and complementary transcribed genes in breast cancer lymph nodes, *Clin Chem* **48,** 1225-1231 (2002).
16. Zehentner BK, Feng Q, Dillon DC, Xu J, Reynolds L, Roche PC, Bennington A, Zhang X, reed SG, Persing DH, Kiviat NB, and Houghton RL, Molecular and Cellular Markers for Breast Cancer, *Proc Amer Assoc Cancer Res* **43,** 40 (2002).
17. Sabath DE, Palomares M, and Koehler K, Development of a reverse transcripatse chain reaction method to quantify breast cancer cells in the peripheral blood, *Era of Hope Department of Defense Breast Cancer Research Program Meeting* **II,** P24-22 (2002).
18. Reinholz MM and Roche PC, Molecular detection of circulating cancer cells for early diagnosis of breast cancer, *Era of Hope Department of Defense Breast Cancer Research Program Meeting* **II,** P24-19 (2002).

APPENDICES: None